REMARKS

No new matter is introduced by the amendments.

The addition of the recitation "elicits protective antibodies" in claim 1 and claim 16 is supported throughout the specification. For example, at page 4, lines 29-31: "An aspect of the invention is a method of eliciting the production of antibodies in mammals using the β-propionamide-linked polysaccharide-protein conjugates that protect the mammals against infection or disease." Also, the specification discloses data that the claimed conjugates do elicit protective antibodies at Tables 5-7, Opsonophagocytic assays (OP).

The replacement of the recitation "comprising an N-propionated polysaccharide or N-propionated oligosaccharide" with "comprise an N-propionated saccharide" does not introduce new matter. This use of the term "saccharide" is used to simply reflect that claimed conjugates may comprise either oligosaccharides or polysaccharides. Support is provided throughout the specification. For example, at page 7, Section A, "Preparation of the N-acryloylated polysaccharides", the specification states that the starting material may be polysaccharides or oligosaccharides: "Polysaccharide or oligosaccharide may be obtained using base hydrolysis or enzymatic hydrolysis..." (page 7, lines 8-9). Support for the addition of "and wherein the N-propionated saccharide is de-N-acetylated and N-acryloylated at the de-N-acetylated terminus" in claim 1, is found throughout the specification, for example, at page 10, lines 6-14.

The addition of the recitations "at a de-N-acetylated terminus" and "coupling at a β-position of a propionate moeity" in claim 16 are supported, for example, at page 10, lines 6-14 and at Example 2. These amendments were made in order to more clearly describe the claimed invention and add no new matter.

The addition of the term "obtained" to claims 3, 4, 5, 17, and 37 was made in order to respond to the Examiner's concern that the term "derived" is indefinite. Applicants disagree with the Examiner's contention concerning the recitation of "derived" which applicants believe properly and definitely describes applicants' invention. The use of "derived" is appropriate because the polysaccharides which are obtained from cells for use in the conjugates are then subjected to modification by being N-acryloylated at de-N-acetylated terminal groups prior to conjugation to protein. Support for the addition of "obtained" is found at page 7, line 8.

Other changes to the claims were made in response to rejections and objections

made by the Examiner. Detailed responses to these rejections and objections are stated in this Response below. No new matter has been added by these amendments.

The changes to the specification only reflect changes to comply with stylistic norms. Entry of the amendments are respectfully requested.

Response to Specification Objection (Examiner's Action #7(c)):

The specification is objected to because the Examiner contends that the claim recitations "N-propionated polysaccharide" and "N-propionated oligosaccharide" do not appear to have antecedence in the specification. Applicants respectfully disagree.

First of all, the recitations "N-propionated polysaccharide" and "N-propionated oligosaccharide" are used in the claims as originally filed, and thus these recitations are disclosed in the specification. Also, the claim recitations "N-propionated polysaccharide" and "N-propionated oligosaccharide" define the claimed invention with a reasonable degree of clarity and precision. The methods used in the instant application apply for both oligosaccharides and polysaccharides. Support is found throughout the specification. For example, at page 8, lines 25-29, under Section 2 "N-Acryloylation of the Polysaccharide" (i.e., the process of making N-propionated saccharides), the specification recites:

> The alkaline or enzymatic hydrolysis of the polysaccharide or oligosaccharide results in the removal of N-acetyl groups from sialic acid and amino sugar residues of the polysaccharides or oligosaccharides. After hydrolysis, the polysaccharide or oligosaccharide is N-acryloylated to the extent desired by using a variety of acryloylating agents.

Thus, the recitations of "polysaccharide" and "oligosaccharide" are supported for the claimed conjugates and methods.

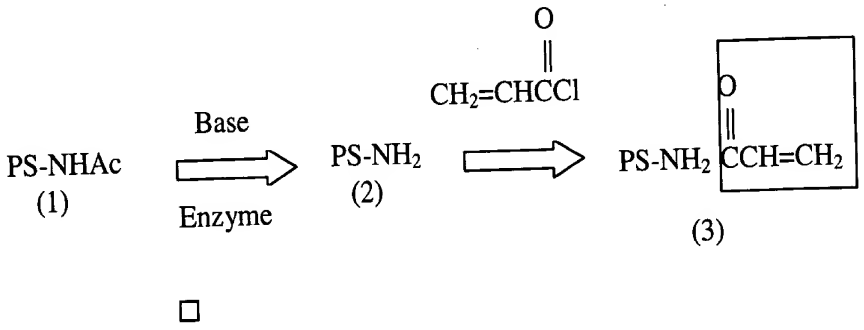
Applicants respectfully disagree with the Examiner's objection to the recitation "N-propionated". First of all, the recitation "N-propionated" is used in the claims as originally filed, and thus the recitation "N-propionated" is disclosed in the specification. Also, the recitations "N-propionated polysaccharide" and "N-propionated oligosaccharide" are defined by a claimed method of forming a immunogenic polysaccharide- or oligosaccharide-protein conjugate. For example, on page 10, lines 6-14, the instant specification states:

> In one method of forming a immunogenic polysaccharide-protein conjugate, an isolated polysaccharide (glycosaminoglycan)

> containing free amino groups or N-acyl groups (e.g. N-acetyl groups) in the sugar residues that constitute its repeating unit, is first treated hydrolyzed using base or enzyme to remove part of all of its N-acyl groups. The free amino groups are then N-acylated with an N-acryloylating reagent to form the N-acryloylated polysaccharide described above. The N-acryloylated polysaccharide is then directly coupled to protein under optimum conditions of pH, temperature and time to form an immunogenic β-propionamido-linked polysaccharide-protein conjugate.

After deacetylation, the free amino groups of a polysaccharide or oligosaccharide are re-N-acylated with an N-acryloylating reagent, as described above, to form the N-acryloylated polysaccharide. This N-acryloylated polysaccharide (oligosaccharide) is equivalently described within the art as an "N-propionated polysaccharide (oligosaccharide)" because the process of N-acryloylation forms a propionate group where the sugar residue has been de-N-acetylated. Again, the methods disclosed in the instant application may be used for <u>both</u> poly- and oligo-saccharides as the specification mentions this fact repeatedly. For example, "This invention provides the ability to produce conjugate molecules wherein the protein is linked to the polysaccharide or oligosaccharide through one or more sites on the polysaccharide or oligosaccharide." (page 9, lines 30-32).

Further support for the process of making N-propionated saccharides can be found in the specification at the paragraph spanning page 10 and page 11, and in Figure 1. Applicants have duplicated Figure 1 on the following page to diagram a claimed method of forming a immunogenic polysaccharide- or oligosaccharide-protein conjugate, so that applicants may show how a polysaccharide (and oligosaccharide) is N-propionated.



In step (1), the polysaccharide is de-acetylated by either base or enzyme, resulting in product (2).

The de-acetylated polysaccharide is then N-acryloylated, so that in (3), the polysaccharide has a propionate group (boxed region in (3)) where originally an acyl group resided (1). Thus, the process of N-acryloylation in the instant invention forms an N-propionated polysaccharide or oligosaccharide. Applicants respectfully request reconsideration and withdrawal of this ground of objection.

Response to Section 112, First Paragraph Rejection (Examiner's Action #8):

Claims 18 and 20 have been rejected because the Examiner contends that the specification does not reasonably provide enablement for a polysaccharide/oligosaccharide-protein conjugate wherein the conjugation is conducted at a pH of 7.0 and in a phosphate buffer.

Specifically, the Examiner refers to various reports in the art (Romanowska *et al.* (1994), Roy *et al.* (1990), Roy *et al.* (1991), and Pon (1992)) as support for her contention that undue experimentation would be required to conjugate an N-acryloylated polysaccharide or oligosaccharide by Michael addition at a pH of about 7.0 in a phosphate buffer medium, because the Examiner contends that the reports cited above show that such conjugation would not work. Thus, the Examiner states "This is important because there is no certainty that this type of conjugation could optimally and/or effectively be conducted at a non-alkaline pH."

The instant specification provides clear guidance as to when conditions of neutral pH should be used so that conjugation would be effective:

> In one embodiment, the method of conjugation is conducted at a pH above 9.0, preferably a pH of about 9.0 to about 10.0 for optimal reactivity of ε-free amino groups of lysine residues on the protein. In another embodiment, the method of conjugation is conducted at a neutral pH of about 7.0 for optimal reactivity of thiol (SH) groups of cysteine residues of the protein. The selection of pH for conducting the method of conjugation may be based on the number of reactive groups in a particular carrier protein. For example, a method using a protein composed of more reactive lysine residues as compared to cysteine residues is preferably conducted at a basic pH. A method of conjugation using a protein composed of more reactive cysteine residues as compared to lysine residues is preferably conducted at about a neutral pH. (page 10, lines 15-25).

The references cited by the Examiner do not mention anything about conjugation of cysteine-rich proteins to polysaccharides. Therefore, applicants respectfully assert that the Examiner's

enablement rejection is improper, since "it is incumbent upon the Patent Office, whenever a rejection on this basis [enablement] is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." (MPEP §2164.04). Applicants respectfully contend that the Examiner has not provided a basis for which to reject the truth or accuracy of the specification's guidance for the conjugation of cysteine-rich proteins at neutral pH.

As for conjugation in phosphate buffers, the Examiner's basis of enablement rejection is also unfounded. Although Romanowska *et al.* states "There was only very slow coupling in phosphate buffers," (page 101), applicants respectfully point out that there was coupling nevertheless. Accordingly, applicants respectfully request reconsideration and removal of this ground of rejection.

Response to Section 112, First Paragraph Rejection (Examiner's Action #9):

Claims 25 and 40 have been rejected under 35 U.S.C. §112, first paragraph, because the Examiner contends that the specification does not enable a pharmaceutical composition or vaccine comprising more than one vaccine component. In particular, the Examiner argues that there is no showing by the specification that combination vaccines comprising an N-propionated polysaccharide or oligosaccharide protein-conjugate would effectively elicit an optimal immune response. The Examiner relies upon two references, Barrington *et al.* (*Infect. Immun.* 61:432-438, 1993) and Corbel (*Biologicals* 22:353-360, 1994) to support her contention that the art reports "potential interference by one or more added vaccine components and suppression of antibody response to the polysaccharide or the carrier protein". Therefore the Examiner argues that undue experimentation would be required by one of ordinary skill in the art to practice the invention as claimed in claims 25 and 40.

Applicants respectfully disagree with the grounds of this rejection. The Examiner asserts that combination vaccines may lead to epitope suppression of anti-polysaccharide responses and therefore claims 25 and 40 would require undue experimentation to practice the invention as claimed. However, this assertion is highly debated in the art, and that combination vaccines have been used repeatedly in the art with great success. For example, Pichichero *et al.* (*J. Infect. Dis.* (1999), 180:1390-3; copy enclosed) reports data that would argue against the

contention that epitope suppression occurs with combination vaccines:

> There have been concerns about interference with Hib vaccine responses, manifested as a decrease in the anti-Hib-PS antibody level, when Hib and DTaP vaccines are combined. We recently studied a DTaP-PRP-T-HB combination vaccine and found evidence that immunologic memory was induced even in infants with lower (<1.0 μg/mL) and even undetectable (<0.10 μg/mL) postprimary anti-Hib-PS antibody levels. Thus, we suggested that the combination vaccine primed the infant immune system for anamnestic anti-Hib-PS antibody responses. Affinity maturation is another major feature of immunologic memory. Here, we showed that DTaP-PRP-T-HB vaccines elicit a high avidity IgG antibody against the Hib PS antigen. Unexpectedly, avidity increased most significantly in the 3-7 months after primary DTaP-PRP-T-HB vaccination, with a marginal further rise after a CRM_{197}-OS booster. (page 1392, first paragraph under Discussion section).

In addition, Goldblatt *et al.* (*J. Infect. Dis.* (1999), 180:538-41; copy enclosed), reports that despite reduced immunogenicity, DTaP-Hib combination vaccines appear to prime for immunologic memory. Thus, the article by N. Halsey, ("Combination Vaccines: Defining and Addressing Current Safety Concerns", *Clinical Infectious Diseases,* (2001), 33(Suppl 4):S312-8; copy enclosed), states: "Historical problems with vaccines, including intussus-ception after rotavirus vaccine, carrier supression with tetanus toxoid conjugate vaccines, and decreased immunogenicity of some *Haemophilus influenzae* type b conjugate vaccines when mixed with acellular pertussis-diphtheria-tetanus, have contributed to some misperceptions about current vaccines. There is no evidence that adding additional vaccines through combination products increases the burden on the immune system, which has the capability of responding to many millions of antigens" (see Abstract).

In addition, applicants bring to the attention of the Examiner, In re Anderson, 176 USPQ 3331 (CCPA 1973). In this case, claims to a laminated dressing wherein the primary layer contains a medicament were rejected under 35 U.S.C. 112, first paragraph, as broader than the enabling disclosure because the term "medicament" was not limited to operative or suitable embodiments. However, the court reversed the rejection, holding that common sense would lead one of ordinary skill in the art to use operable embodiments:

> The concept of medicament or medication involves a highly technical subject in an art requiring a high degree of technical skill – doctors of medicine and pharmacologists. It is common knowledge

> that some medicines of great utility are lethal when used in the wrong quantity, that one man's medicine is another man's poison, and that what is good medicine in one place may be bad medicine in another. The board, seemingly, is demanding a claim limitation to operative medicaments in operative quantity. We think that dependent claims such as the above, which merely add a limitation to the two-layer combination dressing by calling for medication in the primary layer, are inherently limited – by common sense if nothing else –to such medication as would be useful in the particular application. No one of ordinary skill in the art would use any other kind of medicament and there is no practical way to restrict the claim language so as to exclude all inoperative or deletrious medicaments other than by the addition of such redundant terms as "suitable" or "operative for the purposes described."...We are here dealing with combination claims, not with claims for medicaments per se. It is always possible to put something into a combination to render it inoperative. It is not the function of claims to exclude all such matters but to point out what the combination is. (471 F.2d 1237; 176 USPQ (BNA) 331; CCPA (1973)).

Claims 25 and 40 are combination claims of either a pharmaceutical or vaccine composition where the claims add a further element by reciting that the compositions further comprise additional components selected from the group consisting of DTP, DTaP, Td, DTaP-Hib, and DTaP-IPV-Hib. Although the publications cited by the Examiner report some concerns regarding combination vaccines, other combination vaccines are efficacious and widely used, and the Examiner has failed to provide any specific evidence challenging the utility of applicants' claimed invention.

In sum, the clinical significance of epitope suppression is still unclear in the art. However, the specification does provide guidance as to how to determine whether vaccines can elicit the production of antibodies that would be protective, i.e., by opsonophagocytic assays (page 23). These assays will determine whether antibodies elicited by a vaccination, including vaccinations with combined vaccines, are bactericidal and thus protective. Thus, applicants assert that claims 25 and 40 are dependent claims that specify further elements for N-propionated polysaccharide/oligosacharide-protein conjugate combined vaccine and pharmaceutical compositions; and that the specification enables one skilled in the art to determine whether such compositions may elicit bactericidal antibodies and thus warrant further "operative" clinical testing.

Response to Section 112, First Paragraph Rejection (Examiner's Action #10):

Claims 37-40 have been rejected under 35 U.S.C. §112, first paragraph, because the Examiner contends that the specification does not reasonably provide enablement for conjugate vaccines comprising N-acryloylated polysaccharide or oligosaccharide that provide protective immunity against any disease causing organism or cell. Applicants have amended claim 37 such that claims 37-40 now pertain to conjugate vaccines comprising N-acryloylated polysaccharides or oligosaccharides that provide protective immunity against at least one member of the genus of the organism from which the polysaccharide or oligosaccharide component of the polysaccharide-protein conjugate or oligosaccharide-protein conjugate was obtained. Applicants respectfully request reconsideration and removal of this rejection.

Response to Section 112, Second Paragraph Rejection (Examiner's Action #11(a-i)):

(a) Claims 1-5, 8-28 and 38-40 have been rejected under 35 U.S.C. §112, second paragraph, as being indefinite, for failing to particularly point out and distinctly claim subject matter.

Specifically, claims 1 and 16 have been rejected because the Examiner considers the recitation "directly conjugated to" or "directly conjugated" as unclear as to what direct conjugation encompasses.

The recitations "directly conjugated to" or "directly conjugated" refer to conjugations where no spacer or any such molecule is used to couple the polysaccharide or oligosaccharide to the protein. To clarify the application in this regard, applicants have amended the claims so that recitations of "direct conjugation" or "directly conjugated", now state "direct coupling" or "directly coupled". Applicants respectfully request withdrawal of this ground of rejection.

(b) Claims 2-5, 8 and 11-13 have been rejected for lacking proper antecedence for the recitation "A polysaccharide-protein conjugate according to claim...". Applicants have followed the Examiner's suggestion, and have amended the claims to recite –The polysaccharide-protein conjugate according to claim...--. Applicants respectfully request withdrawal of this ground of

rejection.

(c) Claims 38-40 have been rejected for lacking proper antecedence for the recitation "A vaccine according to claim...". Applicants have amended the recitation according to the Examiner's suggestion: --The vaccine according to claim...--. Applicants respectfully request withdrawal of this ground of rejection.

(d) Claims 3-5 and 17 have been rejected because the Examiner contends that the term "derived" is vague and indefinite. Applicants have amended the claims by adding the term "obtained" in order to clarify the meaning of "derived". As stated earlier, "obtained" encompasses, for example, extraction, separation and purification; and "derived" encompasses, for example, modification. Applicants respectfully request withdrawal of this ground of rejection.

(e) Claim 20 has been rejected because the Examiner states that the recitation "carbonate/bicarbonate buffer" is unclear as to what this limitation encompasses. Applicants have amended claim 20 so that the recitation now states "bicarbonate buffer". Applicants respectfully request withdrawal of this ground of objection.

(f) Claim 24 has been rejected because the claim contains incorrect Markush language. Claim 24 has been amended to address the Examiner's concern. Applicants respectfully request withdrawal of this ground of objection.

(g) Claims 25 and 40 have been rejected because these claims have abbreviations that were unspecified in their full terminology. Claim 25 has been amended so that full terminology is used with the abbreviations retained in parenthesis. Applicants respectfully request withdrawal of this ground of objection.

(h) Claims 5 and 15 have been rejected for the inconsistent recitations of "type III" and "serotype III". Applicants have amended claim 5 to recite "type III" in order to maintain consistency with claim 15. Applicants respectfully request withdrawal of this ground of

objection.

(i) Claims 9, 10, 14 and 18-28 have been rejected because of the vagueness of the base claim, claim 1. Applicants have amended claim 1 to address the Examiner's concerns. Applicants respectfully request withdrawal of this ground of objection.

Response to Section 102(b) Rejection, (Examiner's Action #13):

Claims 1-4, 8, 11-14, 22 and 26-28 have been rejected under 35 U.S.C. §102(b) as being anticipated by Roy *et al.* (*J. Chem. Soc. Chem. Commun.* 264-265, 1993), "Roy *et al.*, (1993)". The Examiner contends that Roy *et al.* (1993) reports a polysaccharide conjugate vaccine comprising an N-acryloylated mono- and poly-alpha-(2,8)-sialic acid or colominic acid antigen directly conjugated to a protein. Although applicants respectfully disagree with this rejection, applicants have amended the claims in order to more distinctly claim the instant invention.

All of the elements of amended claim 1 do not read upon the reference cited by the Examiner. Claim 1 encompasses a polysaccharide-protein conjugate or oligosaccharide-protein conjugate comprising an N-propionated saccharide (polysaccharide or oligosaccharide) directly conjugated (i.e., coupled) to a protein at the β-position of a propionate moiety. Roy *et al.* (1993) differs from the instant invention because Roy *et al.* reports conjugations that occur through the reducing end of carbohydrates: "The conjugations of the poly-α-(2,8)-sialic acid **4** to poly-L-lysine **3** and to the protein carriers were accomplished through its reducing end after derivatization to an *N*-acrylamide functionality." (p. 264, emphasis added). In contrast, the claimed invention describes direct coupling not at a reducing end after derivatization, but coupling at the β-position of a propionate moiety.

In the claimed invention, the propionate moiety is formed on one or more saccharides (i.e., oligosaccharides or polysaccharides) by de-acetylation followed by N-acryloylation at the same terminus that was de-acetylated (or "re-N-acryloylation"). Since an oligosaccharide or polysaccharide contains an acetyl group for every sugar residue, the instant invention allows for saccharides to be coupled to multiple protein molecules, or for saccharides to couple to a single protein molecule through multiple sites. The instant specification reveals this novel attribute of the invention, as it states: "The resulting N-acryloylated polysaccharide or

N-acryloylated oligosaccharide is at least about 95% acryloylated or greater" (p. 9, lines 6-7), which allows for the statements, "This invention provides the ability to produce conjugate molecules wherein the protein is linked to the polysaccharide or oligosaccharide through one or more sites on the polysaccharide or oligosaccharide...One or a multiplicity of polysaccharides or oligosacharides may cross-link with one or a multiplicity of protein" (page 9, line 30, to page 10, line 2). In contrast, the conjugates of Roy *et al.* (1993) do not allow multiple couplings because the N-acryloylation occurs at the reducing end of the saccharide.

To further clarify the coupling reaction that may occur for the claimed conjugates, applicants have duplicated Figure 2.11 from the reference: Pon, R.A. (*The Study of Polysialic acid Conjugates.* Master's Thesis, University of Ottawa, pp. 1-251, UMI Dissertation Services, 1992):

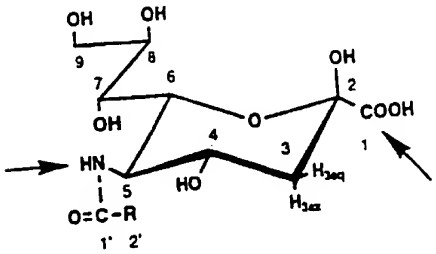


Figure 2.11- Derivatization points of sialic acid.

Depicted above is the repeating unit of colominic acid, where the large arrows represent the termini at which saccharides may be coupled, i.e. "derivatization points". Attached to carbon 5 is an acetyl group. In the instant invention, this acetyl group is de-N-acetylated and re-N-acryloylated, resulting in the formation of a propionate group at carbon 5 (in other repeating sugar residues, the terminus of de-N-acetylation/re-N-acryloylation may occur at a different carbon). This propionate group then serves as the nucleophilic acceptor in the Michael addition-mediated coupling reaction such that a protein is directly coupled to the saccharide at this terminus. Other reported methods of conjugation utilize Michael addition mediated coupling at the reducing terminus, in this example, carbon 1. Therefore, applicants respectfully request reconsideration and removal of this ground of rejection.

Response to Section 102(b) Rejection, (Examiner's Action #14):

Claims 1-3, 14 and 22 have been rejected under 35 U.S.C. §102(b) as being anticipated by Roy *et al.* (*J. Chem. Soc. Chem. Commun.* 536-538, 1991), "Roy *et al.* (1991)". The Examiner contends that Roy *et al.* (1991) reports antigenic carbohydrate protein conjugates comprising synthesized N-acryloylated sugars directly conjugated at the beta position to a lysine-containing protein. Although applicants respectfully disagree with this rejection, applicants have amended the claims in order to more distinctly claim the instant invention.

Roy *et al.* (1991) does not disclose polysaccharide or oligosaccharide-protein conjugates wherein the coupling between the saccharide and protein is direct. Scheme 1, on page 537 of Roy *et al.* (1991), show coupling of a sugar to a protein via a spacer molecule, namely a phenyl group. In contrast, the instant invention claims conjugates wherein the coupling between a saccharide and a protein is direct, without the use of a chemical spacer.

Secondly, Roy *et al.* (1991) does not disclose conjugates wherein the coupling can occur at non-reducing ends of saccharides. Rather, Roy *et al.* (1991) shows that N-acryloylation occurs at the amino group of the phenyl-spacer, and subsequent coupling of the protein occurs at this reducing end. In contrast, the instant invention claim conjugates wherein the coupling occurs at non-reducing ends, namely at de-N-acetylated/re-N-acryloylated termini. Applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Response to Section 102(b) Rejection, (Examiner's Action #15):

Claims 1-4, 8, 11-14, 16, 17 and 19-22 have been rejected under 35 U.S.C. §102(b) as being anticipated by Pon, R.A. (*The Study of Polysialic acid Conjugates.* Master's Thesis, University of Ottawa, pp. 1-251, UMI Dissertation Services, 1992). The Examiner contends that Pon reports polysaccharide or oligosaccharide-protein conjugates produced by a method comprising de-N-acetylating saccharides using a de-N-acetylating base reagent, followed by N-acryloylating the de-N-acetylated saccharide with an acryloylating reagent, and directly conjugating the resultant saccharide to a protein. Although applicants respectfully disagree with this rejection, applicants have amended the claims in order to more distinctly claim the instant invention.

Pon reports the conjugation of N-acryloyl colominic acid onto BSA or IgG,

wherein the colominic acid is 15% N-acryloylated. For example, at page 181, Pon states: "BSA (4-17) (5 mg) or IgG (4-36) (5 mg) was combined with 15% N-acryloylated colominic acid (4-16) (10 mg) and dissolved in 200 µl borate buffer (0.1M; pH 8.3)." However, Pon does not report that this conjugate can stimulate a productive response. Because the sugar is only 15% acryloylated, the degree of protein coupling is limited and will therefore negatively impact the effectiveness of an immune response.

In contrast, the conjugates of the instant application are highly acryloylated and have been shown to produce productive immune responses. For instance, the specification states the degree of acryloylation of the claimed N-propionated saccharides: "The resulting N-acryloylated polysaccharide or N-acryloylated oligosaccharide is at least about 95% acryloylated or greater." (page 9, first paragraph). Also, the specification discloses the immunogenicity of β-propionamido-linked polysaccharide-protein conjugates in Tables 5-8 (pages 24-27), as productive immune responses are observed by ELISA and opsonophagocytic assays. Therefore, applicants have amended the claims by adding the recitation "elicits protective antibodies", in order to more explicity show the advantages and novelty of the instant invention. Applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Response to Section 102(b) Rejection, (Examiner's Action #16):

Claims 1-3, 8, 11-14 and 22 have been rejected under 35 U.S.C. §102(b) as being anticipated by Roy *et al.* (*J. Chem. Soc. Chem. Commun.* 1709-1711, 1990), "Roy *et al.* (1990)". The Examiner contends claims 1-3, 8, 11-14 and 22 have been anticipated because Roy *et al.* (1990) reports conjugates comprising N-acryloylated sialic acid- and sialyloligosaccharide-protein lactoside directly conjugated to proteins by Michael addition. Although applicants respectfully disagree with this ground of rejection, applicants have amended the claims in order to more distinctly claim the novelty of the present invention.

Claims 1-3, 8, 11-14 and 22 are not anticipated by Roy *et al.* (1990) because the present invention relates to polysaccharide- and oligosaccharide-protein conjugates, wherein the formation of these conjugates is dependent upon direct coupling of saccharides and proteins by Michael addition at non-reducing termini. The coupling at the non-reducing termini is contingent upon the de-acetylation and re-N-acryloylation at the same terminus that was de-acetylated. The de-acetylation and re-N-acryloylation results in a propionate group at the

terminus that originally had a acetyl group, and the propionate moiety acts as a nucleophilic acceptor in the Michael addition reaction that couples the saccharide to a protein. In contrast, Roy *et al.* (1990) reports coupling between saccharides and proteins where the coupling occurs at the reducing termini of saccharides (see Scheme 3). Applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Response to Section 102(b) Rejection, (Examiner's Action #17):

Claims 1-3, 8, 11-14 and 22 have been rejected under 35 U.S.C. §102(b) as being anticipated by Romanowska *et al.* (*Methods in Enzymol.* 242:90-101, 1994). The Examiner contends that Romanowska *et al.* reports artificial N-acryloylated sialic acid, sialoside and a T antigen derivative directly conjugated to BSA, tetanus toxoid or poly(L-lysine) via epsilon amino groups.

Although Romanowska reports conjugation of proteins onto N-acryloylamido substituted glycosides, Romanowska does not disclose that such conjugations may occur on non-reducing termini. The instant invention claims conjugations wherein the coupling of protein and saccharide occurs at propionamido termini, where these termini have been de-N-acetylated and re-N-acryloylated. Therefore, the claims have been amended to more distinctly claim the instant invention by making clear that the coupling does not occur at reducing termini. Applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Response to Section 102(b) Rejection, (Examiner's Action #18):

Claims 1-4, 11-14 and 22 have been rejected under 35 U.S.C. §102(b) as being anticipated by Auzanneau *et al.* (*Bioorg. Medicinal Chem.* 4:2003-2010, 1996). The Examiner has rejected these claims because Auzannneau *et al.* reports N-acryloylated Group A streptococcal cell wall oligosaccharide conjugated to BSA or OVA by the addition of ε-amino groups of lysines present on protein. Applicants respectfully disagree with this ground of rejection.

Applicants have amended the claims to clarify the novelty of the instant invention. The claimed conjugates are formed by a distinct process: de-acetylation, N-acryloylation at the termini that are de-acetylated (thereby forming a propionate moiety at these termini), and direct coupling of proteins, via Michael addition, to the β–position of the

propionate moiety(ies). In contrast, Auzanneau *et al.* reports conjugation wherein coupling of protein and saccharide occurs at N-acryloylated termini that have not been de-acetylated (see Figures on 2004). Applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Response to Section 102(b) Rejection, (Examiner's Action #19):

Claims 1-3, 8 and 11-14 have been rejected under 35 U.S.C. §102(b) as being anticipated by Roy *et al.* (*Bioorg. Medicinal chem. Lett.* 2:911-914, 1992), "Roy *et al.* (1992)". The Examiner contends that claims 1-3, 8 and 11-14 are anticipated because Roy *et al.* (1992) reports neoglycoproteins comprising N-acryloylated carbohydrate T antigen, or a blood group trisaccharide determinant conjugated by Michael addition to a protein carrier.

Applicants respectfully disagree with this ground of rejection, as applicants' amendments to the claims have clarified that the present invention pertains to conjugates that are formed by the direct coupling of proteins to propionate groups at non-reducing termini of saccharides, where these propionate groups have been formed by de-acetylation and re-N-acryloylation. In contrast, Roy *et al.* (1992) report indirect coupling, i.e. via a spacer, of proteins to the reducing end of saccharides. Therefore applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Response to Section 103(a) Rejections, (Examiner's Actions #21 and #22):

Claims 1 and 8-10 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Pon, R.A. (*The Study of Polysialic acid Conjugates.* Master's Thesis, University of Ottawa, pp. 1-251, UMI Dissertation Services, 1992) in view of Blake *et al.* (U.S. Patent No. 5,439,808). The Examiner contends that the combination of Pon and Blake *et al.* makes obvious saccharide-protein conjugates, wherein the protein is a *N. meningitidis* outer membrane protein.

Claims 1, 16 and 22-24 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Pon, R.A. (*The Study of Polysialic acid Conjugates.* Master's Thesis, University of Ottawa, pp. 1-251, UMI Dissertation Services, 1992) in view of Blake *et al.* (U.S. Patent No. 5,439,808). The Examiner contends that the combination of Pon and Blake *et al.* makes obvious pharmaceutical compositions comprising saccharide-protein conjugates and

adjuvants.

Applicants respectfully disagree with these grounds of rejection because Pon does not teach or suggest all the claim limitations. As stated previously, Pon reports the conjugation of N-acryloyl colominic acid to BSA or IgG, wherein the colominic acid is 15% N-acryloylated. However, Pon does not report that this conjugate can stimulate a productive response. Because the sugar is only 15% acryloylated, the degree of protein coupling is limited and will therefore negatively impact the effectiveness of an immune response.

In contrast, the conjugates of the instant application are highly acryloylated and have been shown to produce productive immune responses. For instance, the specification states the degree of acryloylation of the claimed N-propionated saccharides: "The resulting N-acryloylated polysaccharide or N-acryloylated oligosaccharide is at least about 95% acryloylated or greater." (page 9, first paragraph). Also, the specification discloses the immunogenicity of β-propionamido-linked polysaccharide-protein conjugates in Tables 5-8 (pages 24-27), as productive immune responses are observed by ELISA and opsonophagocytic assays. Therefore, applicants have amended the claims by adding the recitation "elicts protective antibodies", in order to more explicity show the advantages and novelty of the instant invention.

Thus, the combination of Pon in view of Blake *et al.* does not teach or suggest all of the limitations of the claimed invention. Applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Response to Objections, (Examiner's Actions #23(a)-(g)):

(a) Claims 37 and 38 have been objected to due the recitation "disease causing organism". This recitation has been deleted from the claims.

(b) Claims 5 and 8 have been objected to for inconsistent recitation of "Group B streptococcus" and "group B *Streptococcus*". Claims 5 and 8 have been amended to recite "group B *Streptococcus*".

(c) Claim 15 has been objected to for inconsistent recitations as in (b). Claim 15 has been amended as in (b).

(d) Claim 26 has been objected to for the incorrect recitation "A immunogen". Claim 26 has been amended to recite "An immunogen".

(e) Claims 22 and 26 have been objected to with regard to the recitation "claims 1 or

16". Claims 22 and 26 have been amended to recite "any one of claim 1 or claim 16".

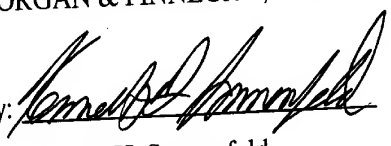
(f) Claim 5 has been objected to for being dependent from a rejected claim (claim 1). Claim 1 has been amended.

(g) Claim 15 has been objected to for including one or more non-elected inventions. Claim 15 is further objected to for lacking antecedent basis in the specification for the limitation: "N-propionated". Claim 15 has been amended to eliminate inclusion of non-elected inventions. In reference to the objection of "N-propionated", please refer to applicants' response to Examiner's action #7(c)). Applicants respectfully request reconsideration and withdrawal of the above grounds of objection.

AUTHORIZATION

No additional fee is believed to be necessary. The Commissioner is hereby authorized to charge any additional fees which may be required for this amendment, or credit any overpayment to Deposit Account No. 13-4500, Order No. 3842-4043US1. A DUPLICATE COPY OF THIS PAGE IS ATTACHED.

Respectfully submitted,
MORGAN & FINNEGAN, L.L.P.

By: 
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APPENDIX:

VERSION OF THE AMENDMENTS TO THE CLAIMS AND SPECIFICATION SHOWING DELETIONS AND ADDITIONS

IN THE CLAIMS:

Claims 1-5, 8, 11-22, 24-26, and 37-40 have been amended as follows:

1. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate that elicits protective antibodies wherein said conjugates [comprising] comprise [an N-propionated polysaccharide or N-propionated oligosaccharide] an N-propionated saccharide directly [conjugated] coupled to a protein at [the] a β-position of [the] a propionate moiety, and wherein the N-propionated saccharide is de-N-acetylated and N-acryloylated at the de-N-acetylated terminus.

2. [A polysaccharide-protein conjugate] The conjugates according to claim 1 wherein the protein comprises at least one lysine or cysteine residue.

3. [A polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 1 wherein the [polysaccharide or oligosaccharide] saccharide is derived from a polysaccharide obtained from bacteria, yeast, cancer cells, or is chemically synthesized.

4. [A polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 1 wherein the [polysaccharide or oligosaccharide] saccharide is derived from a polysaccharide obtained from *Escherichia coli*, Meningococcus, Pneumococcus, Streptococcus, Haemophilus, Neisseria, Salmonella, Klebsiella, or Pseudomonas.

5. [A polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 1 wherein the [polysaccharide or oligosaccharide] saccharide is derived from a polysaccharide obtained [G]group B [streptococcus] *Streptococcus* selected from

the group consisting of [sero]type Ia, [sero]type Ib, [sero]type II, [sero]type III, [sero]type V, [sero]type VIII, and combinations thereof.

8. [A polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 1 wherein the protein is selected from the group consisting of tetanus toxoid, diphtheria toxoid, a *Neisseria meningitidis* outer membrane protein, pneumolysoid, C-β protein from group B *Streptococcus* and non-IgA-binding C-ß protein from group B *Streptococcus*.

9. [The polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 8 wherein the protein is recombinantly produced.

10. [The polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 9 wherein the protein is recombinant *N. meningitidis* outer membrane protein.

11. [A polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 1 wherein the [polysaccharide or oligosaccharide] saccharide comprises a glycosaminoglycan.

12. [A polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 1 wherein the [polysaccharide or oligosaccharide] saccharide comprises glycosyl residues of a structural repeating unit having at least one free amino group or N-acyl group.

13. [A polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 12 wherein the glycosyl residue is selected from the group consisting of glucosamine, galactosamine, mannosamine, fucosamine and sialic acid.

14. The [polysaccharide-protein conjugate or oligosaccharide-protein conjugate] conjugates according to claim 1 wherein the N-propionated [polysaccharide or N-propionated

oligosaccharide] saccharide is directly [conjugated] coupled to an ε-free amino group of a lysine residue or a thiol group of a cysteine residue of the protein.

15. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate comprising [N-propionated *Streptococcus pneumoniae* type 14 polysaccharide-tetanus toxoid conjugate,] N-propionated [G]group B [streptococcus] a *Streptococcus* type III polysaccharide-tetanus toxoid conjugate[, N-propionated Group B streptococcus type II polysaccharide-tetanus toxoid conjugate, N-propionated *E. coli* K1 polysaccharide-protein conjugate, or N-propionated meningococcal C polysaccharide-tetanus toxoid conjugate].

16. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate that elicits protective antibodies produced by a method comprising:

A) de-N-acetylating an isolated polysaccharide or oligosaccharide using a de-N-acetylating reagent to form a de-N-acetylated polysaccharide or a de-N-acetylated oligosaccharide,

B) N-acryloylating the de-N-acetylated polysaccharide or the de-N-acetylated oligosaccharide at a de-N-acetylated terminus with an acryloylating reagent to form an N-propionated polysaccharide or an N-propionated oligosaccharide, and

C) directly [conjugating] coupling at a β-position of a propionate moiety of the N-propionated polysaccharide or [an] the N-propionated oligosaccharide to a protein to form the polysaccharide-protein conjugate or the oligosaccharide protein conjugate.

17. The [polysaccharide-protein conjugate or oligosaccharide-protein conjugate] conjugates according to claim 16 wherein the polysaccharide or oligosaccharide is obtained [derived] from bacteria, yeast, or cancer cells or is [chemical synthesis] chemically synthesized.

18. The [polysaccharide-protein conjugate or oligosaccharide-protein conjugate] conjugates of claim 16 wherein the [conjugation] coupling is conducted at a pH of about 7.0.

19. The [polysaccharide-protein conjugate or oligosaccharide-protein conjugate] conjugates of claim 16 wherein the [conjugation] coupling is conducted at a pH above 9.

20. The [polysaccharide-protein conjugate or oligosaccharide-protein conjugate] conjugates [of] according to claim 16 wherein the [conjugation] coupling is conducted in a reagent selected from the group consisting of phosphate buffer, [carbonate/]bicarbonate buffer, and borate buffer.

21. The [polysaccharide-protein conjugate or oligosaccharide-protein conjugate of] conjugates according to claim 16 wherein the de-N-acetylating reagent is a base or an enzyme and the acryloylating reagent is selected from the group consisting of N-acryloyl chloride, acryloyl anhydride, acrylic acid and a dehydrating agent.

22. A pharmaceutical composition comprising the [polysaccharide-protein conjugate or oligosaccharide-protein conjugate] conjugates according to any one of claim[s] 1 [or] and claim 16 and a pharmaceutically acceptable carrier.

24. The pharmaceutical composition according to claim 23 wherein the adjuvant is selected from the group consisting of alum [or] and stearyl tyrosine.

25. The pharmaceutical composition according to claim 22 further comprising a second component, said second component selected from the group consisting of diphtheria-tetanus-pertussis (DTP), diphtheria-tetanus-acellular pertussis (DTaP), tetanus-diphtheria (Td), diphtheria-tetanus-acellular pertussis-*Haemophilus influenzae type B* (DTaP-Hib), diphtheria-tetanus-acellular pertussis-inactivated poliovirus-*Haemophilus influenzae type B* (DTaP-IPV-Hib), and combinations thereof.

26. [A] An immunogen comprising the [polysaccharide-protein conjugate or oligosaccharide-protein conjugate] conjugates according to any one of claim[s] 1 [or] and claim 16, said immunogen elicits a polysaccharide-specific or an oligosaccharide-specific immune response.

37. A vaccine comprising the [polysaccharide-protein conjugate or oligosaccharide-

protein conjugate] conjugates according to any one of claim 1 [or] and claim 16, wherein said vaccine provides protective immunity against [a disease causing organism or cell] at least one member of a genus of an organism from which the polysaccharide or oligosaccharide component of the polysaccharide-protein conjugate or oligosaccharide-protein conjugate was extracted.

38. [A] The vaccine according to claim 37 wherein the [disease causing] organism [or cell] is selected from the group consisting of bacteria[,] and yeast[, and cancer cell].

39. [A] The vaccine according to claim 38 wherein the bacteria is selected from the group consisting of *Escherichia coli*, Meningococcus, Pneumococcus, Streptococcus, Haemophilus, Neisseria, Salmonella, Klebsiella, [or] and Pseudomonas.

40. [A] The vaccine according to claim 37 further comprising a second immunogen in combination with the polysacharide-protein conjugate or oligosaccharide-protein conjugate, said second immunogen selected from the group consisting of DTP, DTaP, Td, DTaP, Hib, DTaP-IPV-Hib and combinations thereof.

IN THE SPECIFICATION:

The paragraph beginning on page 8, line 30, and ending on page 9, line 7, has been amended as follows:

In one embodiment, the method comprises adding an acryloylating reagent to N-acrylolate an N-deacetylated polysaccharide or oligosaccharide. Examples of acryloylation reagents include but are not limited to acryloyl chloride, acryloyl anhydride, acrylic acid and a dehydrating agent such as dicyclohexylcarbodiimide (DCC), $CH_2CHCOCN$ the like, used in excess at a concentration of about 1 M. In a method of N-acryloylation of an N-deacetylated polysaccharide, the pH is adjusted and maintained at about 9 to about 11, preferably about pH 10 during the reaction. The temperature during reaction is about 2°C to about 8°C, preferably about

4°C. The reaction is carried out over a period of about 1 hour. The resulting N-acryloylated polysaccharide or N-acryloylated oligosaccharide is at least about 95% acryloylated or greater.

The paragraph on page 17, lines 15-24, has been amended as follows:

To increase its solubility the polysaccharide was first partially depolymerized by sonication. 200 mg of *Pneumococcal polysaccharide* type 14 (Lot NO 2020510, American Type Culture Collection) was dissolved in 20 ml of PBS and sonicated for 4 hours at 0°C with a Branson Sonifier Model 450. The resulting polysaccharide was dialyzed and lyophilized and then sized through a [superdex] SUPERDEX™ 200 column equilibrated with phosphate buffered saline (PBS). Peak fractions were pooled and then dialyzed against d.i. water with [Spectra/Por] SPECTRA/POR® Membrane MWCO:3,500. A yield of 157.5mg solid was obtained after lyophilization. The sonicated polysaccharide had an average molecular weight of about 50,000 as measured by SEC-MALLS with the miniDAWN® (Wyatt Technology Corp., Santa Barbara, CA).

The paragraph on page 17, lines 26-32, has been amended as follows:

100 mg of sized type 14 pneumococcal polysaccharide was dissolved in 10 ml of 2N NaOH and then 10 mg of $NaBH_4$ was added to the reaction mixture. This mixture was heated at 100 °C for one hour and then cooled to room temperature. The N-deacetylated component was dialyzed against d.i. water with a [Spectra/Por] SPECTRA/POR® Membrane MWCO:3,500 and lyophilized to give 84 mg of white solid. The N-deacetylated polysaccharide was analysed by H^1-NMR at 500 MHz and was found to contain less than 5 percent residual N-acetyl groups.

The paragraph on page 18, lines 13-25, has been amended as follows:

22 mg of the type 14 N-acryloylated pneumococcal polysaccharide was dissolved in 1.1 ml of Carbonate/Bicarbonate pH 9.5 buffer. Tetanus toxoid monomer 22 mg was added to the reaction mixture. The reaction mixture was incubated overnight at 37 °C. The progress of the conjugation was analyzed with a Biologic system (Bio-Rad) equipped with a [superose] SUPEROSE® 12 column. Conjugation of polysaccharide to tetanus toxoid was indicated by the

progressive increase in a peak, monitored by measurement of UV absorbance at 280 nm, eluting in the void volume of the column. After conjugation was complete, the solution was neutralized to pH 7 with 0.1N HCl and then dialyzed against PBS. The conjugate was purified by passage over a 1.6x60cm column of [Superdex] SUPERDEX™ 200 PG (Pharmacia) and eluted with PBS containing 0.01% thimerosal. Fractions corresponding to the void-volume peak were pooled. Carbohydrate and protein content in the conjugate were estimated by the phenol-sulfuric assay of Dubois et al. (51)and the Coomassie assay of Bradford (9).

The paragraph on page 19, lines 16-21, has been amended as follows:

300 mg of K1 PS was dissolved in 15 mL of 2.0 N NaOH solution to which 150 mg of sodium borohydride was added. The solution was heated at 110 ° C for 6 hours, cooled down to room temperature and diluted with a 20-fold volume of dionized water. After diafiltation through an [Amicon] AMICON™ YM3 membrane with deionized water, the solution was lyophilized yielding 255 mg of N-deacetylated K1 PS. H^1-NMR at 500 MHz confirmed that complete N-deacetylation occurred.

The paragraph beginning on page 19, line 23, and ending on page 20, line 9, has been amended as follows:

To a 10 mL deionized water solution containing 250 mg of de-N-acetylated K1 PS, cooled in an ice bath, was added dropwise acryloyl chloride (Aldrich, Milwaukee,WI) solution, prepared by combining 1 mL of acryloyl chloride with a 1 mL of dioxane. The pH of the solution was maintained between 7.0 and 10.5 by the addition of 2 N sodium hydroxide solution. After completion of the addition, the pH was raised to 13 and maintained between 12.9 to 13.1 for 1 hour at room temperature. The pH of the solution was adjusted to 9.5 by the dropwise addition of 1 N HCL. The solution was diafiltrated with an [Amicon] AMICON™ YM3 membrane in a stircell with deionized water. The retentate was lyophilized to dryness, and the material (N-Acryloyl K1 PS) was stored at in a desiccator in a -20 C freezer. H-NMR at 500 MHz indicated that complete N-acryloylation took place during the reaction.

The paragraph on page 20, lines 11-19, has been amended as follows:

A solution containing 8.4 mg of N-Acryloyl K1 PS and 4.0 mg of recombinant *Neisseria meningitidis* PorB in 0.3 mL of 0.2 M borate, 0.05% [Zwittergen] ZWITTERGENT™ 3,14 (Boehringer Mannhein) pH 9.5 was incubated at 37° C for 3 days. The conjugate was purified by size exclusion chromatography through a [Superdex] SUPERDEX™ 200 preparative grade column, and eluted with PBS containing 0.01% thimerosal. The fractions of uv-280 nm active signal eluting at or close to the void volume of the column were pooled and stored in the refrigerator. The conjugate was analysed for sialic acid and protein content by the resorcinol and Coomassie protein assays respectively.

The paragraph beginning on page 20 line 23, and ending on page 21, line 2, has been amended as follows:

To one ml of rPorB porin solution at a conc of 10 mg/ml in 0.25 M HEPES buffer of pH 8.5 containing 0.25 M sodium chloride and 0.05% [zwittergent] ZWITTERGENT™ 3-14 was added 0.2 ml of 0.05 M N-succinimidyl 3-[2-pyridyldithio]propionate solution. The solution was mixed well and allowed to sit at RT for one hour. To the solution was added 0.06 ml of 1 M dithiothreitol solution in the same buffer. The solution was again mixed well and allowed to sit at RT for an additional two hours. The solution was diluted with 1.3 ml of 0.25 M HEPES buffer of pH 7.0 containing 0.25 M sodium chloride and 0.05% [zwittergent] ZWITTERGENT™ 3-14 and loaded onto a Pharmacia PD-10 desalting column which had been pre-equilibrated with the same buffer. The column was eluted with the same buffer, and eluate was collected and concentrated with an [Amicon Centricon] AMICON™ CENTRICON® 30 concentrator at 5,000 RPM for one hour. The retentate was collected and the protein concentration determined.

The paragraph on page 21, lines 6-12, has been amended as follows:

To 0.17 ml of thiolated rPorB solution at a concentration of 25 mg/ml from above was added 9 mg of N-acryloylated K1 polysaccharide. The solution was mixed well and incubated in an oven of 37° C for 18 hours. The solution was purified through a [Superdex] SUPERDEX™ 200 column (Pharmacia) with PBS as eluent. UV-280-nm-active fractions eluted at or close to the void volume of the column were combined. Analyses showed that the conjugate contained 25 ug/ml of polysaccharide and 188 ug/ml of protein.

The paragraph beginning on page 22, line 19, and ending on page 23, line 21, has been amended as follows:

Immunoassays: Serum antibody to each polysaccharide conjugate was measured by ELISA. The human serum albumin (HSA) (Sigma, St Louis, MO) conjugates used for ELISA assays were prepared by reductive amination. The oxidized polysaccharides were added to HSA followed by reductive amination with NaCNBH3. The conjugates were isolated by gel filtration chromatography, and stored freezed-dried at -70 °C. PS-specific antibody titers were determined by an ELISA as follows. Polystyrene, 96-well, flat-bottom microtiter plates (NUNC™ Polysorb) ([Nunc] NUNC™, Naperville, IL) were coated with PS-HSA conjugates in PBS (0.01 M sodium phosphate, 0.15 M NaCL, pH 7.5) at 0.25 µg/well (100µL/ well) by incubating for 1 hour at 37 °C, followed by a PBS-[Tween] TWEEN™ (0.05% v/v [Tween] TWEEN™ 20 in PBS) wash (5 times). All subsequent incubations were conducted at room temperature. PBS-[Tween] TWEEN™ was used for all required washes. The coated plates were then blocked with PBS-BSA (0.5% w/v bovine serum albumin in PBS) for IgG ELISAs or 0.1% w/v Carnation nonfat dry milk for IgM ELISAs at 0.15 mL / well for 1 hour, followed by a wash. Sera were diluted 2-fold, in duplicate, in the plate at 100 µL/ well and incubated for 1 hour, followed by a wash. Antibody conjugate (peroxidase-labelled goat anti-mouse (Kirkegaard & Perry Lab, Gaithersburg, MD) was added at 100 µL/ well and incubated for 30 minutes, followed by a wash. A 1:1 dye and substrate solution (Kirkegaard & Perry TMB) and peroxide was added at 0.05mL/ well and incubated for 10 minutes. The peroxidase reaction was then stopped with 1 M H_3PO_4 at 0.05 mL/ well, and the plate was read on a [Molecular Devices Emax] MOLECULAR DEVICES™ EMAX® microplate reader ([Molecular Devices] MOLECULAR DEVICES™, Menlo Park, CA) at a wavelength of 450 nm, using 650 nm as a reference wavelength.

Background absorbances were determined in several no-serum control wells and averaged for each plate. For each serum dilution, the average background absorbance was substracted, and then duplicate serum absorbance values were averaged. A modified Scatchard plot was used for the subsequent data analysis, where the absorbance (y-axis) was plotted against the absorbance times the reciprocal dilution (x-axis) (ref). Under conditions allowing equilibrium and antibody excess, a straight line was obtained for each serum dilution series; this line was extrapolated to the x-axis for the determination of an antibody titer. A positive control serum, with a previously determined antibody titer, was used on each plate in order to provide a reference to which all sera were standardized, minimizing plate to plate and day to day variations. The results of these assays are shown in Tables 5, 6 and 7.